

# A $\text{Ca}^{2+}$ Transport System Associated with the Plasma Membrane of *Dictyostelium discoideum* Is Activated by Different Chemoattractant Receptors

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**Abstract.** Amebae of *Dictyostelium* exhibit a transient uptake of extracellular  $\text{Ca}^{2+}$   $\sim 5$  s after activation of surface folate or cAMP receptors (Bumann, J., B. Wurster, and D. Malchow. 1984. *J. Cell Biol.* 98:173–178). To further characterize these  $\text{Ca}^{2+}$  entry systems, we analyzed  $^{45}\text{Ca}^{2+}$  uptake by resting and activated amebae. Like the surface chemoreceptors, folate- and cAMP-induced  $\text{Ca}^{2+}$  uptake responses were developmentally regulated; the former response was evident in vegetative but not aggregation-competent cells, whereas the latter response displayed the opposite pattern of expression. In contrast, other characteristics of these  $\text{Ca}^{2+}$ -uptake pathways were remarkably similar. Both systems (a) exhibited comparable kinetic properties, (b) displayed a high specificity for  $\text{Ca}^{2+}$ , and (c) were inhibited effectively by Ruthenium Red, sodium azide, and carbonylcyanide *m*-chlorophenyl-

hydrazone. These results, together with the finding that vegetative cells transformed with a plasmid expressing the surface cAMP receptor exhibit a cAMP-induced  $\text{Ca}^{2+}$  uptake, suggest that different chemoreceptors activate a single  $\text{Ca}^{2+}$  entry pathway. Additional pharmacological and ion competition studies indicated that receptor-mediated  $\text{Ca}^{2+}$  entry probably does not involve a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger or voltage-activated channels. Chemoattractant binding appears to generate intracellular signals that induce activation and adaptation of the  $\text{Ca}^{2+}$ -uptake response. Analysis of putative signaling mutants suggests that  $\text{Ca}^{2+}$  entry is not regulated by the guanine nucleotide-binding (G) protein subunits  $\text{G}\alpha 1$  or  $\text{G}\alpha 2$ , or by G protein-mediated changes in intracellular cAMP or guanosine 3',5'-cyclic monophosphate (cGMP).

**I**N many higher eukaryotic cells, hormones and neurotransmitters induce changes in cytosolic free  $\text{Ca}^{2+}$  by promoting mobilization of sequestered intracellular  $\text{Ca}^{2+}$  and/or by stimulating entry of extracellular  $\text{Ca}^{2+}$  across the plasmalemma.  $\text{Ca}^{2+}$  mobilization responses can be mediated by increases in cytosolic  $\text{Ca}^{2+}$  (18) or by agonist-induced production of inositol 1,4,5-trisphosphate, which releases  $\text{Ca}^{2+}$  from the ER (4) and other cellular compartments (27, 48). Enhanced uptake of extracellular  $\text{Ca}^{2+}$  occurs following activation of voltage-regulated (40) or ligand-gated (3)  $\text{Ca}^{2+}$  channels, and likely serves a crucial role both in  $\text{Ca}^{2+}$  signaling events and in replenishing agonist-depleted stores (for review, see reference 22). Importantly, many of these  $\text{Ca}^{2+}$ -uptake systems, both voltage-dependent and voltage-independent, appear to be regulated by extracellular factors (for review, see reference 43). In addition, certain of these  $\text{Ca}^{2+}$  channels appear to be modulated directly by guanine nucleotide-binding protein (G protein)<sup>1</sup> subunits while others are regulated indirectly by second messengers such as

cAMP, guanosine 3',5'-cyclic monophosphate (cGMP), and inositol polyphosphates (43).

In the lower eukaryote, *Dictyostelium discoideum*, chemoattractant-induced changes in the concentration of free cytosolic  $\text{Ca}^{2+}$  are probably important in the regulation of certain cellular processes during development (see reference 35). Growing amebae feed on bacteria, and respond chemotactically to folate, a compound secreted by the bacteria (39). Upon starvation, these cells aggregate into multicellular structures, which undergo morphogenesis and differentiation to form fruiting bodies (for review, see reference 30). Cell aggregation and differentiation are regulated, in part, by endogenously generated waves of extracellular cAMP. Activation of the cAMP or folate chemoreceptors induce a number of cellular events including a rapid increase in intracellular cGMP (51), and an influx of extracellular  $\text{Ca}^{2+}$  (7, 16, 50). Relatively little is known about the properties or the nature of chemoattractant-mediated  $\text{Ca}^{2+}$  uptake systems in *Dictyostelium*. Moreover, it is unclear whether the folate and cAMP receptors couple with the same or different  $\text{Ca}^{2+}$  transport systems. To characterize further receptor-activated  $\text{Ca}^{2+}$  entry in this organism, we developed a  $^{45}\text{Ca}^{2+}$  assay system to measure accurately uptake of

1. Abbreviations used in this paper: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; G protein, guanine nucleotide-binding protein.

low micromolar concentrations of  $\text{Ca}^{2+}$  into resting and chemoattractant-stimulated cells. Our results suggest that vegetative and aggregation-competent amoebae of *Dictyostelium* possess a highly specific  $\text{Ca}^{2+}$  uptake system that is regulated by distinct chemoreceptors.

## Materials and Methods

### Materials

Materials used and their sources were as follows:  $^{45}\text{CaCl}_2$  (14.7 mCi/mg  $\text{Ca}^{2+}$ ; 1 Ci = 37 GBq) and [2,8- $^3\text{H}$ ]cAMP (27 Ci/mmol) (ICN Biomedicals, St. Laurent, Canada); NCS tissue solubilizer (Amersham Corp., Oakville, Canada);  $\text{CaCl}_2$  standard (Orion Research Inc., Cambridge, MA); cAMP, DTT, methoxyverapamil (D-600), 3,4,5-trimethoxybenzoate 8-(diethylamino)octyl ester-HCl (TMB-8), nifedipine, and nifedipine (Sigma Chemical Co., St. Louis, MO); folate,  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , and  $\text{MnCl}_2 \cdot \text{H}_2\text{O}$ , (BDH Inc., Toronto, Canada);  $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ , and Ruthenium Red (Aldrich Chemical Co., Milwaukee, WI); sodium azide (J. T. Baker Chemical Co., Phillipsburg, NJ); DMSO (Fisher Scientific, Unionville, Canada); Geneticin (Gibco/BRL, Burlington, Canada). All other chemicals were of analytical grade and were obtained from the suppliers indicated in references 35, 36.

### Strains and Culture Conditions

The following aggregation-competent, haploid strains of *D. discoideum* were used in this study: HC91 and HC6 (10), XP55 and NP368 (41), AX2 (49), and AX3 cells transformed with the plasmid pBS18B6, carrying a cloned cAMP receptor gene (cAR1 cells) or the plasmid pBS18, lacking the receptor sequence (BS18 cells) (26; provided by P. N. Devreotes [Johns Hopkins School of Medicine, Baltimore, MD]). Several other AX3 derivatives were used:  $\text{GalS}$ , a transformant cell line overexpressing  $\text{Gal}$  (28), JH131, a null mutant of the  $\text{G}_1$   $\alpha$ -subunit and JH130, a control transformant (provided by J. Hadwiger and R. A. Firtel [University of California, San Diego, CA]), and JH104, a null mutant of the  $\text{G}_2$   $\alpha$ -subunit (provided by J. Hadwiger, R. A. Firtel, and P. N. Devreotes). Two classes of aggregation-deficient strains were also used: a Synag mutant, HC347 (PD7-2-2) (32), and two Frigid A mutants, HC85 and HC112 (12). All cell lines, except AX2, cAR1, BS18,  $\text{GalS}$ , and JH104 were grown in association with *Klebsiella aerogenes* on SM agar plates at 22°C as described (10). The other strains were grown axenically in liquid HL-5 medium as described (35), except that the growth medium for cAR1, BS18, and  $\text{GalS}$  was supplemented with 20  $\mu\text{g}$  of Geneticin/ml.

To obtain aggregation-competent amoebae, vegetative cells were permitted to differentiate on nonnutrient agar at 7°C for 16 h (37). The plates were then transferred to 22°C for 1–2 h before the cells were harvested and used in  $\text{Ca}^{2+}$  uptake experiments. In some experiments, amoebae of strains JH131,  $\text{GalS}$ , JH104, HC85, and PD7-2-2 were treated with exogenous cAMP pulses as described (29).

### $\text{Ca}^{2+}$ Uptake Assay

Unless indicated otherwise, all  $\text{Ca}^{2+}$  uptake studies were performed with amoebae of strain HC91. To prepare cells for uptake experiments, amoebae at the desired developmental stage were harvested by centrifugation (700 g, 2 min, 22°C) in H buffer (20 mM Hepes/KOH, 5 mM KCl, pH 7.0) and washed twice in the same buffer. The amoebae were then resuspended to a concentration of  $1 \times 10^8$  cells/ml in H buffer and shaken (22°C) for 10 min at 250 rpm on a gyrotory shaker (model G76; New Brunswick Instruments, Edison, NJ).

In most experiments,  $\text{Ca}^{2+}$  uptake into resting amoebae was initiated by adding 100  $\mu\text{l}$  of cell suspension to a microcentrifuge tube containing 100  $\mu\text{l}$  of an uptake medium (22°C) composed of 20 mM Hepes/KOH, 5 mM KCl, 100  $\mu\text{M}$   $\text{CaCl}_2$ , and  $\sim 0.5 \mu\text{Ci}$   $^{45}\text{CaCl}_2$  (pH 7.0). To measure  $\text{Ca}^{2+}$  uptake into folate- or cAMP-stimulated cells, the uptake medium was supplemented with 40  $\mu\text{M}$  folate or 2  $\mu\text{M}$  cAMP, respectively. At the times indicated,  $^{45}\text{Ca}^{2+}$  entry was terminated by the addition of 100  $\mu\text{l}$  of ice-cold H buffer containing 775 mM  $\text{CaCl}_2$ . The cell suspensions were centrifuged immediately at 12,000 rpm for 4 s in an Eppendorf model 5414 centrifuge (Eppendorf Gerätebau, Hamburg, FRG), and the supernatants were discarded. The cell pellets were then resuspended in 1 ml of ice-cold H buffer containing 10 mM  $\text{CaCl}_2$ , recentrifuged, solubilized in NCS and counted as described (11). Nonspecific  $\text{Ca}^{2+}$  binding was determined by adding

cells to uptake medium containing 225 mM  $\text{CaCl}_2$ . Chemoattractant-induced  $\text{Ca}^{2+}$  uptake at each timepoint was determined by subtracting the amount of  $\text{Ca}^{2+}$  taken up by resting cells from the amount accumulated by the stimulated cells. Protein was measured by the method of Lowry et al. (31) using BSA as a standard.

In certain experiments,  $\text{Ca}^{2+}$  uptake was followed in the presence of putative inhibitors of  $\text{Ca}^{2+}$  transport. Stock solutions of these compounds (10–40 mM) were prepared in DMSO (methoxyverapamil, nifedipine, nifedipine, and carbonylcyanide *m*-chlorophenylhydrazone [CCCP]) or H buffer (all other compounds), and stored at  $-20^\circ\text{C}$ . The DMSO concentration in the assay system never exceeded 1%; this concentration had no effect on the  $\text{Ca}^{2+}$  uptake response.

### cAMP-binding Assay

Amoebae were harvested, washed twice by centrifugation in 10 mM  $\text{Na}_2\text{-HPO}_4/\text{KH}_2\text{PO}_4$  buffer (pH 6.5), and resuspended in the same buffer to give a concentration of  $2 \times 10^8$  cells/ml. Binding of 100 nM [ $^3\text{H}$ ]cAMP to the cells was determined in triplicate using the ammonium sulfate assay (45).

## Results

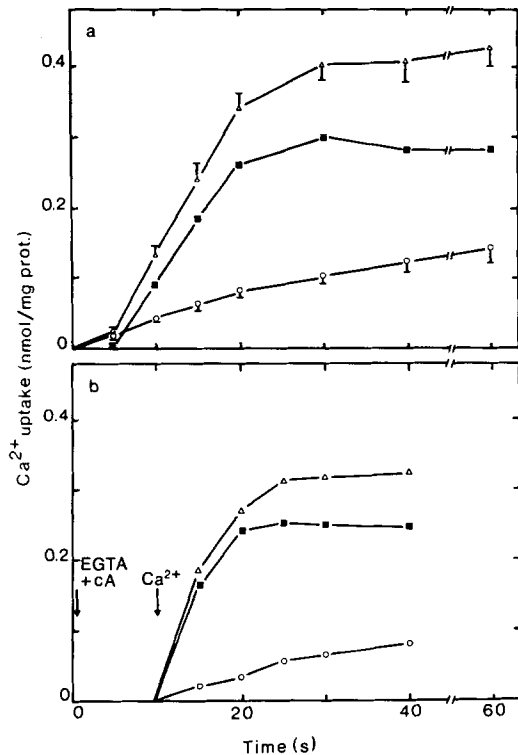
### Identification of Folate- and cAMP-stimulated $\text{Ca}^{2+}$ Uptake

Fig. 1 *a* shows the kinetics of  $\text{Ca}^{2+}$  accumulation by suspensions of aggregation-competent amoebae of strain HC91 in the presence or absence of cAMP. Amoebae treated with cAMP initially took up  $\text{Ca}^{2+}$  at the same rate as nonstimulated cells. However, after a delay of  $5.5 \pm 0.4$  s (mean  $\pm$  SEM,  $n = 5$ ),  $\text{Ca}^{2+}$  uptake by the stimulated cells increased sharply for 20–25 s, and then ceased. Similar results were obtained with amoebae of strain AX2 grown axenically (data not shown). When the cAMP receptor was activated 10 s before the addition of  $\text{Ca}^{2+}$ , uptake proceeded immediately at a high rate. The time at which the response terminated, however, was unchanged, i.e., 25–30 s after addition of stimulus (Fig. 1 *b*). In growth-phase cells, folate stimulated  $\text{Ca}^{2+}$  uptake with kinetics very similar to those observed for the cAMP-mediated response of developing cells, except the delay preceding  $\text{Ca}^{2+}$  entry was  $8.5 \pm 0.3$  s (mean  $\pm$  SEM,  $n = 7$ ) (data not shown).

The developmental regulation of folate- and cAMP-induced  $\text{Ca}^{2+}$  influx is illustrated in Fig. 2. When cells were starved on nonnutrient agar, folate-mediated uptake was maximal in vegetative cells and remained constant for  $\sim 4$  h before declining steadily to undetectable levels by 10 h. Under similar conditions, cAMP-activated  $\text{Ca}^{2+}$  uptake was barely detectable during the first 4 h of development. The magnitude of this response increased dramatically between 4 and 9 h as the cells became fully aggregation competent, and then declined slightly over the next 5 h. The developmental regulation of folate-induced  $\text{Ca}^{2+}$  uptake was markedly different when cells were permitted to develop in suspension. Under these conditions, the response was maximal during the first 0.5 h of starvation, and then declined to low levels by 3.5 h.

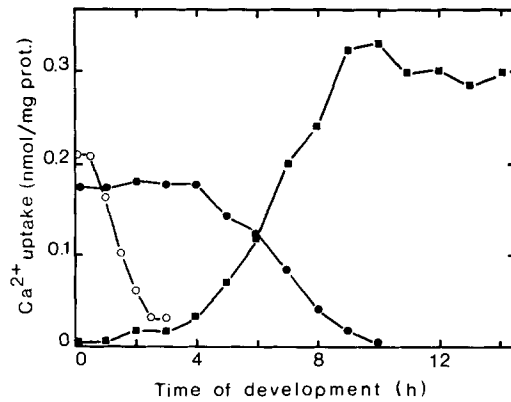
### Chemoattractants Alter the Kinetic Properties of $\text{Ca}^{2+}$ Influx

The amount of  $\text{Ca}^{2+}$  accumulated by aggregation-competent amoebae depended upon the concentration of the cAMP stimulus (Fig. 3 *a*). Stimuli of 1 nM failed to induce a detectable response while 10  $\mu\text{M}$  cAMP elicited maximal uptake ( $302 \pm 12$  pmol  $\text{Ca}^{2+}$  transported/ $10^7$  cells; mean  $\pm$  SEM,



**Figure 1.** Time course of cAMP-induced Ca<sup>2+</sup> uptake into amebae of strain HC91. (a) Basal or cAMP-stimulated Ca<sup>2+</sup> uptake into aggregation-competent cells was assayed under standard conditions as described in Materials and Methods. (b) At zero time, aggregation-competent cells ( $1 \times 10^7$ ) were added to a modified uptake medium consisting of (final concentrations) 20 mM HEPES/KOH, 5 mM KCl, 20  $\mu$ M EGTA (pH 7.0) and to an identical medium containing 1  $\mu$ M cAMP. After 10 s, the free Ca<sup>2+</sup> concentration was adjusted to 50  $\mu$ M by the addition of <sup>45</sup>CaCl<sub>2</sub>. The amount of <sup>45</sup>Ca<sup>2+</sup> added was calculated by means of a computer program based on Fabiato and Fabiato (19). At the times indicated, Ca<sup>2+</sup> uptake was terminated and the samples were processed as described in Materials and Methods. Values are shown for Ca<sup>2+</sup> uptake into resting (○) and cAMP-stimulated (Δ) cells, and for cAMP-induced uptake (■). Each point is the mean of data obtained in five to six (a) or two (b) independent experiments. In a, bars represent SEM.

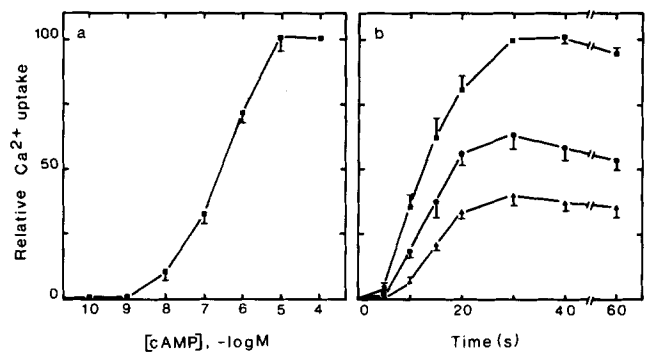
$n = 3$ ). Half-maximal uptake occurred at 280 nM cAMP. The dose-response profile was not altered by the presence of 10 mM DTT, a compound that inhibits cyclic nucleotide phosphodiesterase activity in *Dictyostelium* (24). Also, a similar dose-response profile was obtained with axenically grown amebae of strain AX2 (data not shown). Concentrations of cAMP between 100 nM and 100  $\mu$ M had no effect on the time of initiation or termination of Ca<sup>2+</sup> uptake (Fig. 3 b); therefore, stimulus concentration appears to influence the rate, rather than the duration, of the response. To determine if cAMP alters the affinity of the uptake system for external Ca<sup>2+</sup>, initial rates of Ca<sup>2+</sup> entry into resting and cAMP-stimulated cells were determined at extracellular free Ca<sup>2+</sup> concentrations ranging from 10 to 400  $\mu$ M (Fig. 4 a). For nonstimulated cells, the initial rates of Ca<sup>2+</sup> uptake increased slowly over the entire Ca<sup>2+</sup> concentration range examined. In contrast, the rates of uptake into cAMP-stimulated cells increased sharply at external Ca<sup>2+</sup> concentrations



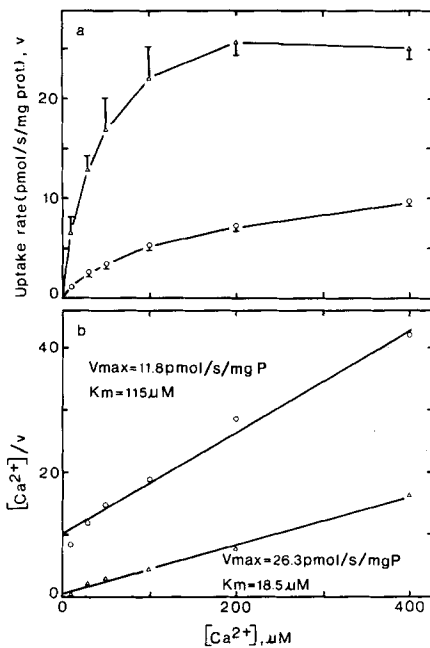
**Figure 2.** Developmental regulation of folate- and cAMP-stimulated Ca<sup>2+</sup> uptake. Cells were permitted to develop at 22°C on phosphate-buffered agar as described by Coukell et al. (13). At the times indicated, cells were harvested, washed, and assayed for 30 s for folate- (●) and cAMP- (■) stimulated Ca<sup>2+</sup> uptake as described in Materials and Methods, except that the stimulus was 100  $\mu$ M. (○) Vegetative cells ( $1 \times 10^8$ /ml) were shaken in H buffer for the times indicated and then assayed for 30 s for folate-induced Ca<sup>2+</sup> uptake as described in Materials and Methods. Values shown are the means of data from two experiments.

up to 100  $\mu$ M, and then remained constant at concentrations >200  $\mu$ M. In Fig. 4 b, the same data are plotted according to Hanes (23). The results suggest that Ca<sup>2+</sup> uptake follows Michaelis-Menten kinetics both in the presence and absence of a cAMP stimulus. However, addition of cAMP increases the  $V_{max}$  of Ca<sup>2+</sup> transport approximately twofold and lowers the apparent  $K_m$  for Ca<sup>2+</sup> approximately sixfold.

Ca<sup>2+</sup> uptake by vegetative cells was influenced in a similar fashion by the addition of folate. Half-maximal and maximal levels of receptor-stimulated Ca<sup>2+</sup> influx occurred at 135 nM and 10  $\mu$ M folate, respectively (data not shown). The Ca<sup>2+</sup> transport system(s) of resting and folate-stimulated vegetative cells also exhibited Michaelis-Menten kinet-



**Figure 3.** Effect of cAMP concentration on the magnitude (a) and time course (b) of cAMP-mediated Ca<sup>2+</sup> uptake. Aggregation-competent cells were assayed for cAMP-dependent Ca<sup>2+</sup> uptake as described in Materials and Methods except that in (a) uptake was followed for 30 s in the presence of 0.1 nM to 100  $\mu$ M cAMP, and, in b, the assay system contained 100 nM (▲), 1  $\mu$ M (●), or 100  $\mu$ M (■) cAMP. cAMP-induced Ca<sup>2+</sup> uptake values are expressed relative to the 30 s timepoint value in the presence of 100  $\mu$ M cAMP. Each point is the mean  $\pm$  SEM of results obtained in three (a) or four (b) separate experiments.



**Figure 4.** cAMP-induced changes in the kinetic properties of the  $\text{Ca}^{2+}$  transport system of aggregation-competent amebae. (a)  $\text{Ca}^{2+}$  uptake into resting ( $\circ$ ) and cAMP-stimulated amebae ( $\Delta$ ) was measured under standard conditions except that the extracellular  $\text{Ca}^{2+}$  concentration was varied from 10 to 400  $\mu\text{M}$ . To determine initial rates of  $\text{Ca}^{2+}$  entry, uptake was terminated 10, 15, and 20 s after the start of the reaction. Values are the means  $\pm$  SEM of results from three independent experiments. (b) A Hanes plot of the data from a. The lines were fitted by regression analysis.

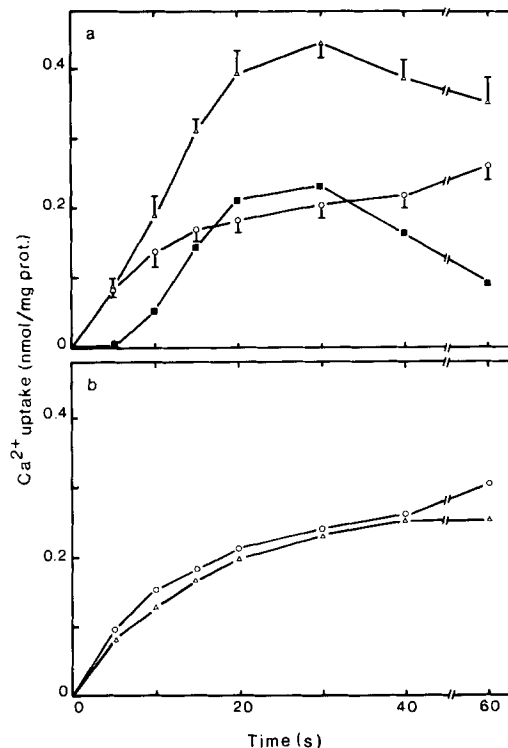
ics. Folate increased the  $V_{\text{max}}$  of uptake 1.5-fold (from 15.5 to 23.3 pmol  $\text{Ca}^{2+}$  transported/s per mg protein) and reduced the apparent  $K_m$  for external  $\text{Ca}^{2+}$   $\sim 7.5$ -fold (from 125 to 16.9  $\mu\text{M}$ ) (data not shown).

#### A Single $\text{Ca}^{2+}$ Entry Pathway Appears to Couple with Both the Folate and cAMP Receptors

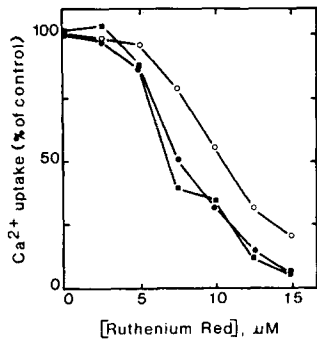
The similar profiles of folate- and cAMP-stimulated  $\text{Ca}^{2+}$  uptake, together with the observations that the two chemoattractants induce comparable changes in the kinetic properties of  $\text{Ca}^{2+}$  influx, suggested that the different chemoreceptors might couple to the same  $\text{Ca}^{2+}$  transport system. To investigate this possibility,  $\text{Ca}^{2+}$  uptake was analyzed in growth-phase AX3 cells transformed with a plasmid carrying a gene encoding a surface cAMP receptor (cAR1 cells). These amebae expressed  $8.9 \pm 1.3$ -fold (mean  $\pm$  SEM,  $n = 6$ ; range 6.1–15.2-fold) higher levels of surface cAMP-binding sites than cells transformed with the same plasmid minus the receptor gene (BS18 cells). As observed with aggregation-competent wild-type cells, cAMP elicited a rapid influx of external  $\text{Ca}^{2+}$  into vegetative amebae expressing cAR1 (Fig. 5 a). With these cells, the cAMP-induced  $\text{Ca}^{2+}$  uptake began after a delay of  $6.8 \pm 0.9$  s (mean  $\pm$  SEM,  $n = 5$ ) and continued for 15–20 s. However, unlike aggregation-competent wild-type cells,  $\text{Ca}^{2+}$  accumulation by the cAR1 transformants decreased after  $\sim 30$  s (compare Figs. 1 and 5 a). This decline in cellular  $\text{Ca}^{2+}$  is not due to a loss of the uptake response over the course of the experiment, since cAR1 cells shaken in suspension for

1 h retained high levels of cAMP-induced  $\text{Ca}^{2+}$  uptake (data not shown). Growth-phase BS18 amebae failed to exhibit cAMP-mediated  $\text{Ca}^{2+}$  entry (Fig. 5 b), a finding consistent with the observation that these cells possessed low levels of surface cAMP-binding sites relative to cAR1 cells. Both BS18 and cAR1 vegetative amebae showed a low but reproducible  $\text{Ca}^{2+}$  uptake in response to folate (stimulated uptake was 75–100 pmol  $\text{Ca}^{2+}$ /mg protein 30 s after folate addition).

To compare further the  $\text{Ca}^{2+}$  uptake systems in vegetative and aggregation-competent cells, the effects of various putative inhibitors of  $\text{Ca}^{2+}$  transport were examined. The following  $\text{Ca}^{2+}$ -channel blockers, at the concentrations indicated, had little or no effect on cAMP-stimulated  $\text{Ca}^{2+}$  uptake in aggregation-competent cells: 3,4,5-trimethoxybenzoate 8-(diethylamino)octyl ester-HCl (TMB-8) (50  $\mu\text{M}$ ), verapamil (50  $\mu\text{M}$ ), diltiazem (100  $\mu\text{M}$ ), methoxyverapamil (100  $\mu\text{M}$ ), nifedipine (100  $\mu\text{M}$ ), and nicardipine (100  $\mu\text{M}$ ). In contrast, Ruthenium Red was an effective inhibitor of cAMP-induced  $\text{Ca}^{2+}$  uptake with an  $\text{IC}_{50}$  (i.e., concentration required to reduce  $\text{Ca}^{2+}$  uptake by 50%) of  $\sim 7.5$   $\mu\text{M}$  (Fig. 6). Similar dose-inhibition profiles and  $\text{IC}_{50}$  values (7–10  $\mu\text{M}$ ) were obtained when the effect of this compound was examined on the cAMP-stimulated  $\text{Ca}^{2+}$  uptake by growth-phase cAR1 cells and the folate-induced uptake by vegetative HC91 cells (Fig. 6). cAMP- and folate-stimulated  $\text{Ca}^{2+}$  uptake were also inhibited by sodium azide with  $\text{IC}_{50}$ s



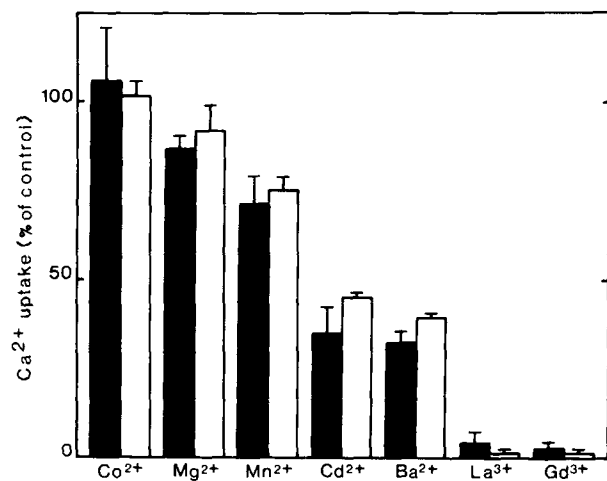
**Figure 5.** Time course of cAMP-stimulated  $\text{Ca}^{2+}$  entry into (a) cAR1 and (b) BS18 amebae. Growth-phase cells were assayed for  $\text{Ca}^{2+}$  uptake as described in Materials and Methods except that uptake was monitored in the presence ( $\Delta$ ) or absence ( $\circ$ ) of 10  $\mu\text{M}$  cAMP. Each point in a is the mean  $\pm$  SEM of results from four experiments. ( $\blacksquare$ ) cAMP-stimulated  $\text{Ca}^{2+}$  uptake. In b,  $\text{Ca}^{2+}$  uptake values are the means of results obtained in two experiments.



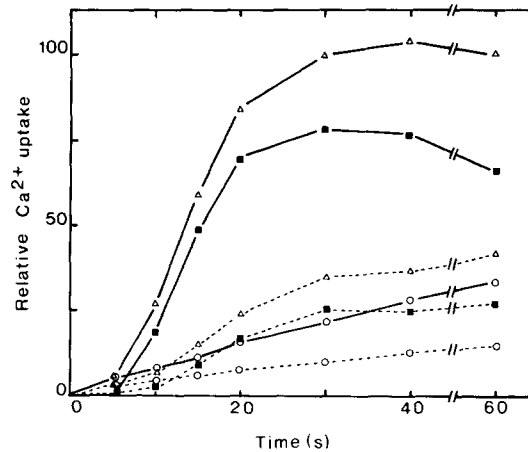
**Figure 6.** Effect of Ruthenium Red on chemoattractant-stimulated  $\text{Ca}^{2+}$  uptake. Folate-induced  $\text{Ca}^{2+}$  entry into vegetative ( $\circ$ ) and cAMP-induced  $\text{Ca}^{2+}$  uptake into aggregation-competent ( $\bullet$ ) HC91 cells were assayed for 30 s as described in Materials and Methods except that the assay system contained  $10 \mu\text{M}$   $\text{Ca}^{2+}$  and various concentrations of Ruthenium Red. cAMP-induced  $\text{Ca}^{2+}$  entry into vegetative

cAR1 cells ( $\blacksquare$ ) was monitored under identical conditions except that the cAMP stimulus was  $10 \mu\text{M}$ . For each profile, results shown are the means of two separate experiments.

of  $\sim 9$  and  $14 \mu\text{M}$ , respectively, and by CCCP ( $\text{IC}_{50} = \sim 2 \mu\text{M}$  in both cases) (data not shown). Ion competition studies were performed to determine if various di- and trivalent cations could reduce folate- and cAMP-mediated  $\text{Ca}^{2+}$  entry. In the presence of  $500 \mu\text{M}$  test ion and  $10 \mu\text{M}$  extracellular  $\text{Ca}^{2+}$ , both  $\text{Ca}^{2+}$ -uptake systems were inhibited poorly by  $\text{Co}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , moderately by  $\text{Cd}^{2+}$  and  $\text{Ba}^{2+}$ , and strongly by  $\text{La}^{3+}$  and  $\text{Gd}^{3+}$  (Fig. 7). Further experiments revealed that  $\text{La}^{3+}$  and  $\text{Gd}^{3+}$  inhibited both folate- and cAMP-mediated  $\text{Ca}^{2+}$  uptake with  $\text{IC}_{50}$ s of 200–250  $\mu\text{M}$  (data not shown). These inhibitor results, together with the finding that vegetative cAR1 (but not BS18) amoebae expressed high levels of surface cAMP receptors and exhibited a cAMP-stimulated  $\text{Ca}^{2+}$  uptake similar to that of aggregation-competent wild-type cells, supports the idea that a single  $\text{Ca}^{2+}$ -uptake system might mediate folate- and cAMP-induced  $\text{Ca}^{2+}$  entry in *Dictyostelium*.



**Figure 7.** Ability of various cations to inhibit folate- and cAMP-mediated  $\text{Ca}^{2+}$  uptake. Folate-stimulated  $\text{Ca}^{2+}$  uptake into vegetative cells (closed bars) and cAMP-induced uptake into aggregation-competent cells (open bars) were measured for 30 s under standard conditions except that the assay system contained  $10 \mu\text{M}$   $\text{Ca}^{2+}$  and  $500 \mu\text{M}$  test cation. Results are expressed relative to the folate- and cAMP-induced  $\text{Ca}^{2+}$  uptake of control cells not receiving test ions. Results shown are the means  $\pm$  SEM of data obtained in three experiments.



**Figure 8.** Effect of sodium azide on the cAMP-stimulated  $\text{Ca}^{2+}$  uptake of aggregation-competent cells.  $\text{Ca}^{2+}$  uptake into resting ( $\circ$ ) or cAMP-stimulated ( $\Delta$ ) amoebae was measured as described in Materials and Methods except that the assay system contained  $10 \mu\text{M}$   $\text{Ca}^{2+}$  (solid lines) or  $10 \mu\text{M}$   $\text{Ca}^{2+}$  and  $15 \mu\text{M}$  sodium azide (dashed lines). ( $\blacksquare$ ) cAMP-stimulated  $\text{Ca}^{2+}$  uptake. Values are expressed relative to the level of  $\text{Ca}^{2+}$  uptake at 30 s by cAMP-stimulated cells not receiving sodium azide, and are the means of results obtained in two experiments.

### Regulation of the Receptor-activated $\text{Ca}^{2+}$ Transport System

The inability of high concentrations of classical voltage-gated  $\text{Ca}^{2+}$  channel blockers to inhibit cAMP-mediated  $\text{Ca}^{2+}$  uptake suggested that this transport system is not regulated directly by changes in membrane potential. This idea was supported further by experiments with sodium azide, a compound reported to rapidly depolarize the plasma membrane of *Dictyostelium* without altering appreciably cellular ATP levels (44). Addition of  $100 \mu\text{M}$  sodium azide to the assay system reduced cAMP-stimulated  $\text{Ca}^{2+}$  uptake by  $\sim 90\%$  (data not shown). To determine if membrane depolarization influences the kinetics of  $\text{Ca}^{2+}$  entry,  $\text{Ca}^{2+}$  uptake was measured in the presence of  $15 \mu\text{M}$  sodium azide, a concentration that reduced  $\text{Ca}^{2+}$  transport by both resting and stimulated cells by 55–65%. Under these conditions, the times at which cAMP-stimulated  $\text{Ca}^{2+}$  uptake commenced and terminated were unchanged (Fig. 8).

To investigate other mechanisms possibly involved in coupling the chemoreceptors to  $\text{Ca}^{2+}$  uptake, a number of putative signal transduction mutants of *Dictyostelium* were analyzed. Recent evidence suggests that the folate and cAMP receptors are coupled to certain effector enzymes via G proteins (21, 25). Therefore, chemoattractant-activated  $\text{Ca}^{2+}$  transport was examined in the Frigid A mutants HC85 and HC112, which are defective in the gene encoding the G protein  $\alpha$ -subunit,  $\text{G}\alpha 2$  (21). Growth-phase amoebae of strains HC85 and HC112 exhibited a folate-stimulated  $\text{Ca}^{2+}$  uptake similar to their parental strains, HC6 and HC91, respectively. When these mutants were starved on nonnutrient agar at  $22^\circ\text{C}$  for 16 h, they failed to show a cAMP-induced  $\text{Ca}^{2+}$  uptake response, but continued to exhibit a strong folate-mediated uptake (data not shown). However, when the HC85 amoebae were pulsed for  $\sim 9$  h with  $50 \text{ nM}$  cAMP, they exhibited an  $8.5 \pm 0.9$ -fold (mean  $\pm$  SEM,  $n = 3$ ; range

6.9–9.6-fold) increase in the levels of surface cAMP-binding sites and a small but reproducible cAMP-induced  $\text{Ca}^{2+}$  uptake (stimulated uptake was 50–90 pmol/mg protein 30 s after cAMP addition). Similar results were obtained using amoebae of strain JH104, a  $\text{G}\alpha 2$ -null mutant (data not shown). In other experiments, the following strains were found to exhibit normal kinetics of chemoreceptor-induced  $\text{Ca}^{2+}$  uptake upon activation with folate and/or cAMP: JH131, a mutant carrying a deletion of the  $\text{G}1$   $\alpha$ -subunit gene;  $\text{G}\alpha 1\text{S}$ , a transformant that expresses 10–20-fold higher levels of  $\text{G}\alpha 1$  than control cells (28); NP368, which possesses elevated cGMP pools (42); and PD7-2-2, which fails to exhibit adenylate cyclase activation in response to a cAMP stimulus (32). In each case, mutant or transformant cell lines were compared with their parent strain or to an appropriate control transformant. Chemoattractant-induced  $\text{Ca}^{2+}$  entry was also not influenced by 5 mM caffeine, a compound reported to block cAMP receptor-induced activation of the adenylate cyclase in *Dictyostelium* (6). Together, these observations suggest that the folate- and cAMP-stimulated  $\text{Ca}^{2+}$  uptake system(s) is not regulated directly by the G protein subunits  $\text{G}\alpha 1$  or  $\text{G}\alpha 2$ , or by changes in the intracellular concentrations of cAMP or cGMP.

## Discussion

To study receptor-activated  $\text{Ca}^{2+}$  entry in *Dictyostelium*, we developed a  $^{45}\text{Ca}^{2+}$ -uptake assay to measure  $\text{Ca}^{2+}$  accumulation by resting and chemoattractant-stimulated cells. The folate- and cAMP-induced  $\text{Ca}^{2+}$  uptake systems detected by this assay are probably the same systems identified previously by others (7, 50). Consistent with the findings of these investigators, responsive cells stimulated with folate or cAMP accumulated  $\text{Ca}^{2+}$  at the same rate as resting cells for 6–9 s, and then exhibited a dramatic influx of  $\text{Ca}^{2+}$ , which continued for 20–25 s. In contrast, Europe-Finner and Newell (16) reported that neither folate nor cAMP enhanced the rates of  $\text{Ca}^{2+}$  uptake into stimulated cells, although they increased the magnitude of  $\text{Ca}^{2+}$  uptake. Chemoattractant-induced increases in rates of  $\text{Ca}^{2+}$  entry likely were obscured in these experiments by the use of  $\text{LaCl}_3$  (100  $\mu\text{M}$ ) to block  $^{45}\text{Ca}^{2+}$  transport. Recent evidence (5; see Results) suggests that this concentration of  $\text{LaCl}_3$  does not effectively inhibit  $\text{Ca}^{2+}$  influx in *Dictyostelium*.

Changes in the levels of folate- and cAMP-induced  $\text{Ca}^{2+}$  uptake during development suggest that these responses are regulated by specific chemoreceptors. For example, when amoebae are starved on nonnutrient agar, the decrease in folate-induced  $\text{Ca}^{2+}$  uptake (Fig. 2) correlates closely with reported changes in the numbers of folate receptors on the surface of these cells and their chemotactic responsiveness to folate (14). Similarly, as the amoebae become aggregation competent, there are parallel increases in cAMP-stimulated  $\text{Ca}^{2+}$  uptake (Fig. 2), the level of cell surface cAMP receptors (34), and chemotactic sensitivity of the cells to cAMP (47).

In agreement with Bumann et al. (7), we observed that maximal folate- and cAMP-induced  $\text{Ca}^{2+}$  uptake occur at a stimulus concentration of 10  $\mu\text{M}$ . Moreover, the dose-response profiles obtained with folate in the two studies are comparable, with  $\text{EC}_{50}$  values of 135–200 nM. However, the cAMP-induced  $\text{Ca}^{2+}$  uptake system characterized in the

present study appears considerably less responsive to low concentrations of cAMP ( $\text{EC}_{50} = 280$  nM) than the transport system described earlier ( $\text{EC}_{50} = 5$  nM). This difference is probably not due to the strains used or to degradation of the cAMP stimulus in our experiments because we obtained the same profile with the strain used in the earlier study (AX2), and with reaction mixtures containing a cyclic nucleotide phosphodiesterase inhibitor (DTT). Interestingly, a dose-response profile, very similar to the one presented here, was reported for the cAMP-induced efflux of  $\text{K}^+$  from *Dictyostelium* cells, a process thought to be regulated by the influx of  $\text{Ca}^{2+}$  (compare Fig. 3 a to Fig. 1 in reference 1).

Analysis of  $\text{Ca}^{2+}$  uptake by vegetative and aggregation-competent cells suggests that the same  $\text{Ca}^{2+}$  transport system(s) is operative at both developmental stages. For example, at each stage, resting cells appear to possess a low-affinity system with comparable kinetic properties for  $\text{Ca}^{2+}$  transport. When the amoebae are stimulated by folate (vegetative) or cAMP (aggregation-competent), both chemoattractants enhance dramatically (6–7.5-fold) the affinity of the  $\text{Ca}^{2+}$  transport system while exerting more modest increases (1.5–2-fold) on maximal rates of  $\text{Ca}^{2+}$  entry. At present, it is uncertain whether the chemoattractants activate the  $\text{Ca}^{2+}$  uptake system detectable in resting amoebae or a second  $\text{Ca}^{2+}$  influx pathway. However, the observations that  $\text{Ca}^{2+}$  uptake into both folate- and cAMP-stimulated cells exhibits linear Michaelis–Menten kinetics support the former model. The idea that the folate and cAMP chemoreceptors couple with the same  $\text{Ca}^{2+}$  uptake system is also suggested by other results. For instance,  $\text{Ca}^{2+}$  uptake responses induced by both chemoattractants (a) exhibit similar time courses, (b) display comparable sensitivities to inhibition by Ruthenium Red ( $\text{IC}_{50} = 7$ –10  $\mu\text{M}$ ), sodium azide ( $\text{IC}_{50} = 9$ –14  $\mu\text{M}$ ) and CCCP ( $\text{IC}_{50} = \sim 2$   $\mu\text{M}$ ), and (c) show the same degree of specificity in the presence of competing multivalent cations (Fig. 7). Finally, growth-phase cAR1 receptor transformants (but not control transformants) express high levels of surface cAMP-binding sites and exhibit cAMP-mediated  $\text{Ca}^{2+}$  uptake with properties (e.g., time course and sensitivity to inhibition by Ruthenium Red) very similar to those of the cAMP-induced  $\text{Ca}^{2+}$  uptake system detectable in aggregation-competent wild-type cells (Figs. 1 a, 5 a, and 6).

What is the nature of the chemoattractant-induced  $\text{Ca}^{2+}$ -uptake system in *Dictyostelium*? It is unlikely that  $\text{Ca}^{2+}$  entry is mediated by a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. When cAMP-induced  $\text{Ca}^{2+}$  uptake was measured in a medium containing 5 mM  $\text{Na}^+$ , the reported intracellular  $\text{Na}^+$  concentration in this organism (33), no reduction in rate of  $\text{Ca}^{2+}$  entry was observed (data not shown). In addition, the  $\text{Ca}^{2+}$  flux measurements of Bumann et al. (8) suggest that a  $\text{H}^+/\text{Ca}^{2+}$  exchanger is not involved. To determine if receptor-activated  $\text{Ca}^{2+}$  uptake in this organism occurs via plasma membrane channels, pharmacological experiments were performed. Treatment of the cells with sodium azide or CCCP, compounds reported to induce membrane depolarization (20, 44), did not change the time course of stimulated  $\text{Ca}^{2+}$  uptake (Fig. 8). This suggests that  $\text{Ca}^{2+}$  uptake does not involve voltage-gated channels. However, these agents did reduce the magnitude of  $\text{Ca}^{2+}$  uptake by both resting and stimulated cells, thus raising the possibility that changes in membrane potential might regulate transport indirectly by altering the electrochemical gradient. Sodium azide and

CCCP inhibited folate- and cAMP-induced  $\text{Ca}^{2+}$  uptake over a very narrow concentration range (data not shown); this supports the idea that these agents depolarize the plasma membrane. Similar results were obtained with Ruthenium Red (Fig. 6). This compound is known to cause membrane depolarization in other systems, but it also blocks  $\text{Ca}^{2+}$  channels and interacts specifically with  $\text{Ca}^{2+}$ -binding proteins (9). Its mechanism of action in *Dictyostelium* remains to be determined. Unexpectedly, cAMP-induced  $\text{Ca}^{2+}$  uptake was insensitive to several classes of organic  $\text{Ca}^{2+}$  channel antagonists including the 1,4-dihydropyridines, (nifedipine, nicardipine), phenylalkylamines (verapamil, methoxyverapamil), and benzothiazepines (diltiazem). In addition, stimulated  $\text{Ca}^{2+}$  influx was inhibited poorly by cations (i.e.,  $\text{La}^{3+}$ ,  $\text{Gd}^{3+}$ ,  $\text{Co}^{2+}$ , and  $\text{Cd}^{2+}$ ) (Fig. 7) known to act as  $\text{Ca}^{2+}$  channel blockers in mammalian systems (38). Since different classes of  $\text{Ca}^{2+}$  channels vary widely in their biophysical and pharmacological properties (2), the inability of these chemicals and ions to block  $\text{Ca}^{2+}$  uptake does not eliminate the possibility that this transport system is a channel. Verification that  $\text{Ca}^{2+}$  channels are involved must await the appropriate electrophysiological experiments.

The kinetics of chemoattractant-mediated  $\text{Ca}^{2+}$  uptake suggest that receptor binding activates both the initiation and termination of  $\text{Ca}^{2+}$  transport. Receptor activation appears to stimulate the rate of  $\text{Ca}^{2+}$  entry (Figs. 1 and 3 b) by generating an intracellular signal(s) that increases the number of active channels (transporters?) and their affinity for  $\text{Ca}^{2+}$  (Fig. 4 b). Since the onset of stimulated  $\text{Ca}^{2+}$  uptake only occurs after a lag of  $>5$  s, even in the presence of saturating stimulus (100  $\mu\text{M}$ ) (Fig. 3 b), the activation process might involve several biochemical steps or a slow process such as phosphorylation. This idea is supported by the observation that when cAMP receptors are activated 10 s before the addition of  $\text{Ca}^{2+}$ , ion influx occurs without a delay (Fig. 1 b). Receptor occupancy also appears to initiate an adaption process which limits the duration of  $\text{Ca}^{2+}$  transport. Stimulated  $\text{Ca}^{2+}$  uptake terminates  $\sim 30$  s after receptor activation regardless of whether the amoebae are treated with suboptimal or saturating levels of cAMP (Fig. 3 b). Chemoattractant-induced activation and adaption have been reported for several other responses in *Dictyostelium* (15, 46).

Recent evidence indicates that both the folate- and cAMP-mediated signal transduction pathways in *Dictyostelium* might be regulated by G proteins (for review see references 21, 25). Interestingly, the cloned cAMP receptor, when expressed in vegetative cells which possess very low levels of endogenous receptor, appears to couple to the chemoattractant-stimulated  $\text{Ca}^{2+}$  transport system (Fig. 5 a). This finding suggests that G proteins might also regulate receptor-mediated  $\text{Ca}^{2+}$  entry. At present, the biochemical components in this pathway are unknown; however, our results seem to eliminate a number of possibilities. First, the G protein  $\alpha$ -subunit,  $\text{G}\alpha 1$ , is unlikely involved since chemoattractant-induced  $\text{Ca}^{2+}$  uptake into a  $\text{G}\alpha 1$ -null mutant and a  $\text{G}\alpha 1$ -over-expressing cell line was similar to that of wild-type cells. Second, analysis of Frigid A mutants and a  $\text{G}\alpha 2$ -disruption mutant suggests that  $\text{G}\alpha 2$  is not required for folate- or cAMP-induced  $\text{Ca}^{2+}$  entry. However, since cAMP-pulsed HC85 and JH104 cells exhibit a weak cAMP-stimulated  $\text{Ca}^{2+}$  uptake, it is possible that  $\text{G}\alpha 2$  is normally involved in the cAMP-activated process, and, in its absence, a pulse-

induced "back-up" G-protein is able to couple the receptor to this  $\text{Ca}^{2+}$  entry system. Third, although G protein-linked signal transduction pathways appear to mediate activation of both adenylate and guanylate cyclases in *Dictyostelium* (25), our results with the signaling mutants NP368 and PD7-2-2 indicate that stimulated  $\text{Ca}^{2+}$  entry is not regulated by changes in either intracellular cGMP or cAMP. Noninvolvement of intracellular cAMP is also suggested by (a) pretreatment of cells with caffeine, a compound which inhibits adenylate cyclase activation (6), does not influence cAMP-induced  $\text{Ca}^{2+}$  uptake, (b) activation of adenylate cyclase by cAMP is slower (51) than the onset of cAMP-stimulated  $\text{Ca}^{2+}$  entry, and (c) folate does not induce production of cAMP in vegetative amoebae (51). In *Dictyostelium*, G proteins also appear to couple the cAMP receptors to the production of inositol polyphosphates (17). It remains to be determined whether these intracellular messengers (or the diacylglycerol/protein kinase C system) are involved in the regulation of receptor-mediated  $\text{Ca}^{2+}$  entry systems in this organism.

We are grateful to Drs. Peter Devreotes, Maureen Pupillo, Richard Firtel, Jeff Hadwiger (Johns Hopkins University School of Medicine), and Akiko Kumagai (University of California, San Diego), for useful discussions and for providing strains used in this study, and to Ms. Anne Cameron for preparation of the figures.

J. L. Milne was a recipient of a Province of Ontario Graduate Scholarship. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

Received for publication 18 June 1990 and in revised form 14 September 1990.

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